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DOI: https://doi.org/10.22203/eCM.v025a23

OSSEOINTEGRATION OF BIOCHEMICALLY MODIFIED IMPLANTS IN AN OSTEOPOROSIS RODENT MODEL

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Abstract

The present study examined the impact of implant surface modifications on osseointegration in an osteoporotic rodent model. Sandblasted, acid-etched titanium implants were either used directly (control) or were further modified by surface conditioning with NaOH or by coating with one of the following active agents: collagen/chondroitin sulphate, simvastatin, or zoledronic acid. Control and modified implants were inserted into the proximal tibia of aged ovariectomised (OVX) osteoporotic rats (n = 32/group). In addition, aged oestrogen competent animals received either control or NaOH conditioned implants. Animals were sacrificed 2 and 4 weeks post-implantation. The excised tibiae were utilised for biomechanical and morphometric readouts (n = 8/group/readout). Biomechanical testing revealed at both time points dramatically reduced osseointegration in the tibia of oestrogen deprived osteoporotic animals compared to intact controls irrespective of NaOH exposure. Consistently, histomorphometric and microCT analyses demonstrated diminished bone-implant contact (BIC), peri-implant bone area (BA), bone volume/tissue volume (BV/TV) and bone-mineral density (BMD) in OVX animals. Surface coating with collagen/chondroitin sulphate had no detectable impact on osseointegration. Interestingly, statin coating resulted in a transient increase in BIC 2 weeks post-implantation; which, however, did not correspond to improvement of biomechanical readouts. Local exposure to zoledronic acid increased BIC, BA, BV/TV and BMD at 4 weeks. Yet this translated only into a non-significant improvement of biomechanical properties. In conclusion, this study presents a rodent model mimicking severely osteoporotic bone. Contrary to the other bioactive agents, locally released zoledronic acid had a positive impact on osseointegration albeit to a lesser extent than reported in less challenging models.

Keywords: Implant; osseointegration; osteoporosis; animal model; surface coating; histomorphometry; biomechanics.

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Introduction

Subsequent to placement of an implant into bone, the osseous wound undergoes sequentially haemostasis, inflammation and proliferation analogous to the phases of fracture healing (Schindeler *et al.*, 2008). Primary implant stability is enabled by an interlock between an implant and the host bone. During osseous healing, host bone in proximity to the implant will be resorbed and new woven bone will be formed (Davies, 1998). This leads to a transient decrease in stability until secondary implant stability is established. Remodelling processes around the implant will continue for an extended time period depending on species, loading of the implant, and bony template (Branemark *et al.*, 1977).

The implant surface structure and composition has an impact on the attachment of proteins and subsequent cellular processes, which are relevant to implant osseointegration (Junker *et al.*, 2009). Ideally, an implant should be characterised by high primary stability and a sufficient and persistent degree of osseointegration (Esposito *et al.*, 1998; Sennerby and Meredith, 2008; Martin *et al.*, 2009). In recent years, several techniques of surface modification emerged in order to stimulate peri-implant bone formation and thus osseointegration. It was demonstrated, that roughened surfaces positively influence biomechanical anchorage and bone formation (Butz *et al.*, 2006). Moreover, surface conditioned implants showed increased rates of bone formation (Buser *et al.*, 2004; Stadlinger *et al.*, 2009b). Another approach, which is increasingly explored, is the coating of implant surfaces with components of the bone extracellular matrix in order to influence mediators involved in early phases of osseointegration (Rammelt *et al.*, 2007; Stadlinger *et al.*, 2009a). Such surface coatings have been shown to increase bone formation in comparison to sandblasted, acid-etched implants in uncompromised host bone (Stadlinger *et al*., 2009a). From the clinical point of view, stimulation of osseointegration is of particular interest in compromised bone. Osteoporosis, characterised by low bone mass and increased bone fragility, is a skeletal

disorder that represents such a compromised setting. The correlation between systemic bone and oral bone loss has been demonstrated (Jeffcoat, 2005; Wactawski-Wende *et al*., 2005), a correlation to implant loss, however, has not been found (Holahan *et al*., 2008). A common therapy for osteoporotic patients is the application of drugs that inhibit bone resorption and thus further bone loss. Bisphosphonates represent the largest group of these anti-resorptive drugs currently in clinical use (Rachner *et al*., 2011). Accordingly, coating of implant surfaces with bisphosphonates has been explored for its ability to improve implant osseointegration and testing in animal studies yielded positive results (Wermelin *et al*., 2007; Wermelin *et al*., 2008a). Another approach is to improve osseointegration by stimulation of bone formation. This has been explored utilising statin coated implants (Moriyama *et al*., 2010), based on the finding that statins induce bone morphogenetic protein-2 (BMP-2) expression and promote osteoblastic BMP signalling (Mundy *et al*., 1999), which is critical during osseointegration and fracture healing. Also for this approach positive findings were reported in a pre-clinical animal model (Du *et al*., 2009).

In the present study we analysed biochemically modified implants in a severely compromised setting. Implants were inserted into the proximal tibia of oestrogen deprived rats with established osteoporosis. The aim of the study was to test whether these biochemical modifications that have been reported previously to enhance osseointegration in healthy bone would yield a similar effect in osteoporotic bone.

Materials and Methods

Animals

The experimental protocol of this study and animal care conformed to the Swiss federal law for animal protection under the control of the Basel-Stadt Cantonal Veterinary Office, Switzerland. Hundred and sixty, six-month old female virgin Wistar rats underwent bilateral ovariectomy (OVX), while another 64 rats underwent sham surgery and remained intact. Fourteen weeks post-surgery the animals were divided into five OVX- and two non-OVX groups (32 rats/group) with an even distribution of proximal tibial bone-mineral density as assessed by microCT and body weight within the OVX groups and the intact groups. Each of the seven groups was randomly divided into two sub-groups (16 rats/sub-group), designated to 2 and 4 weeks of implant healing. The animals received one implant in the left proximal tibia metaphysis. Tibial bone samples from eight animals per sub-group were assessed by microCT and histomorphometry; the additional eight samples were utilised for biomechanical testing.

Implants

Experimental threaded titanium implants (grade 4) with an inner diameter of 1.1 mm, outer diameter of 1.7 mm and a length of 3.0 mm (Thommen Medical AG, Waldenburg, Switzerland) received different surface treatments. Implants had a cuboid implant head in order to allow biomechanical testing. The surface of all implants had been sandblasted and thermally acid-etched (SAE). The S_a value of this procedure is approximately 2.0 µm (Stadlinger *et al*., 2012). Sixty-four implants were conditioned by a solution containing hydroxide ions prior to implant placement as described previously (Stadlinger *et al*., 2012). Thirty-two implants were coated in a solution of 1 mg/mL collagen type I and chondroitin sulphate at a concentration of 50 µg/mL, as described previously (Stadlinger *et al*., 2009a). Another 32 implants were coated with a simvastatin-chitosan complex using a modified spin coating procedure. A homogeneous suspension of 57 µg simvastatin complex in 5 µL acetone containing 35 µg simvastatin per implant has been used. The implants were fixed in a horizontally positioned stirring motor (Eurostar Digital, IKA–Werke, Staufen, Germany) rotating at a constant speed of 300 rpm. The simvastatin suspension was dropped into the implant thread turns using a precision pipette (Eppendorf Reseach, Hamburg, Germany; 0.5-10 µL volume), which was positioned at a distance of 1-2 mm over the rotating implant. Implant rotation results in a homogeneous distribution of the coating within the implant thread.

Finally, 32 implants were coated with a zoledronic acid–stearate complex using the method described above. A homogeneous suspension of 23.8 µg of zoledronic-acid-stearate complex in acetone containing 8.5 µg zoledronic acid per implant was used. After coating, both simvastatin and zoledronate-containing implants were stored for 30 min at 60 °C to remove the solvent. All implants were sterilised by gamma-radiation.

In vitro release studies have been performed measuring the amount of released simvastatin and zoledronic acid, respectively, within a time period of 14 days. For this purpose titanium discs (grade 4, 15 mm in diameter, surface treated in the same way as the implants) coated with the simvastatin-chitosan and the zoledronic acid-stearate complex, respectively, were stored into simulated body fluid (SBF) medium (simvastatin-coated discs) and, physiological NaCl solution (zoledronic acid-coated discs) respectively, and the released amount of bioactive agent after 1, 2, 3, 5, 7 and 14 days was measured. The amount of released simvastatin and zoledronic acid was quantified by UV/Vis spectroscopy. For zoledronic acid quantification the spectrophotometric method reported by Gallez *et al*. (1988) was employed. All experiments were run in duplicates. Release data are shown in Table 1.

Implant procedure

Animals were anaesthetised with ketamine and xylazine by intraperitoneal injection and received buprenorphine for analgesic purposes. All operative procedures were performed under sterile conditions. For implant placement, a 15 mm longitudinal incision was made along the medial side of the tibia and a musculoperiosteal flap was elevated. A conical drill with a diameter of 1.1-1.5 mm in diameter was used to generate, under water cooling, an osteotomy 2 mm distal to the growth plate of the proximal left tibia. Implants were placed using an implant screwdriver. After placement, a rounded polyether ether ketone healing-cap was placed on the cuboid implant top. The soft tissue was repositioned and sutured in two layers, using resorbable sutures (Safil 6x0, Braun, Melsungen,

Table 1. *In vitro* release of simvastatine and zoledronic acid.

Complex	Cumulative amount of released agent [%]					
	Release time [d]					
	1	2	3	5	7	14
Simvastatin-chitosan	20.4 ± 2.8	22.3 ± 3.1	22.7 ± 3.1	22.9 ± 3.1	23.1 ± 3.1	23.2 ± 3.1
Zoledronic acid-stearate	34.2 ± 2.8	49.5 ± 5.5	62.9 ± 6.1	69.5 ± 6.2	71.1 ± 4.7	71.1 ± 4.7

Table 2. Results of microcomputed tomography at two and four weeks (µCT).

Group	Surface	Healing time	BIC cancellous bone			BV/TV 200 µm			BMD 200 µm	
		[weeks]	*n*	[%]	SD	*n*	[%]	SD	*n*	[mgHA/ccm]	SD
non-OVX	reference	2	7	68.6#	10.8	7	48.6#	13.3	7	883.9#	107.5
	reference	4	7	67.7#	13.9	7	50.5#	17.1	7	891.3#	152.3
	conditioned	2	6	69.5#	14.9	7	50.7#	15.7	7	891.0#	140.5
	conditioned	4	6	62.1#	13.7	7	46.6#	17.9	7	861.4#	161.7
OVX	reference	2	6	31.3*	11.9	7	10.8*	5.7	7	491.2*	105.8
	reference	4	6	20.6*	2.4	7	4.7*	3.5	7	361.6*	62.6
	conditioned	2	6	30.2*	10.7	7	11.8*	5.1	8	470.7*	89.6
	conditioned	4	7	25.4*	4.5	7	6.4*	2.4	7	404.5*	38.6
	collagen/CS	2	8	36.3*	6.7	8	12.6*	4.3	8	503.9*	72.8
	collagen/CS	4	7	21.4*	3.8	7	3.8*	1.7	7	355.8*	24.8
	simvastatin	2	6	50.8#	6.6	7	19.3*	6.4	7	592.7*	81.5
	simavstatin	4	6	35.6*	10.7	7	10.5*	5.4	7	453.9*	83.9
	zoledronic acid	2	6	31.8*	8.4	7	15.1*	8.8	7	532.5*	125.7
	zoledronic acid	4	8	47.8*#	10.4	8	23.0*#	11.3	8	602.1*#	128.0

Significantly different ($p \leq 0.05$) compared to reference in non- OVX (*) or OVX (#) rats at the same time point.

Germany). An aerosol bandage was applied (Flint MED, Togal-Werk AG, München, Germany) and wound healing was controlled daily for the first week and twice per week during the following healing periods. All animals received fluorochrome markers by subcutaneous injection 10 days (alizarin complexone, 20 mg/kg; Merck, Zug, Switzerland) and 3 days (calcein, 30 mg/kg, Sigma-Aldrich, Steinheim, Germany) prior to sacrifice.

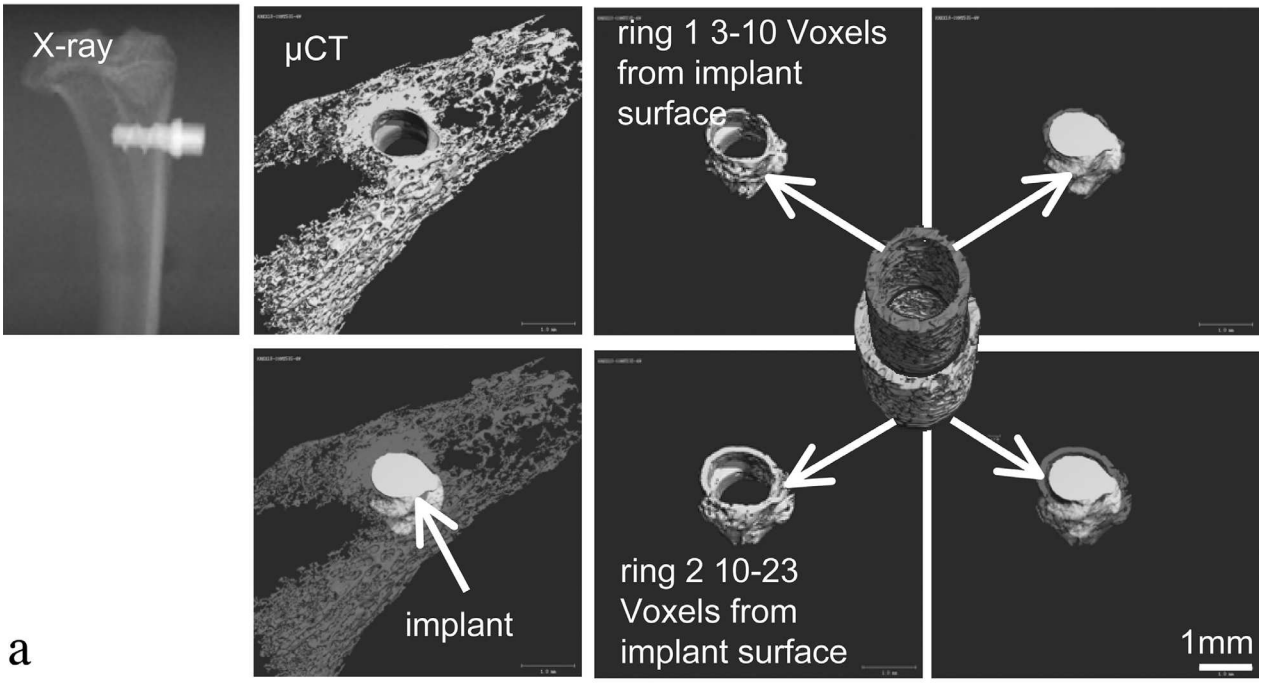
Tissue processing

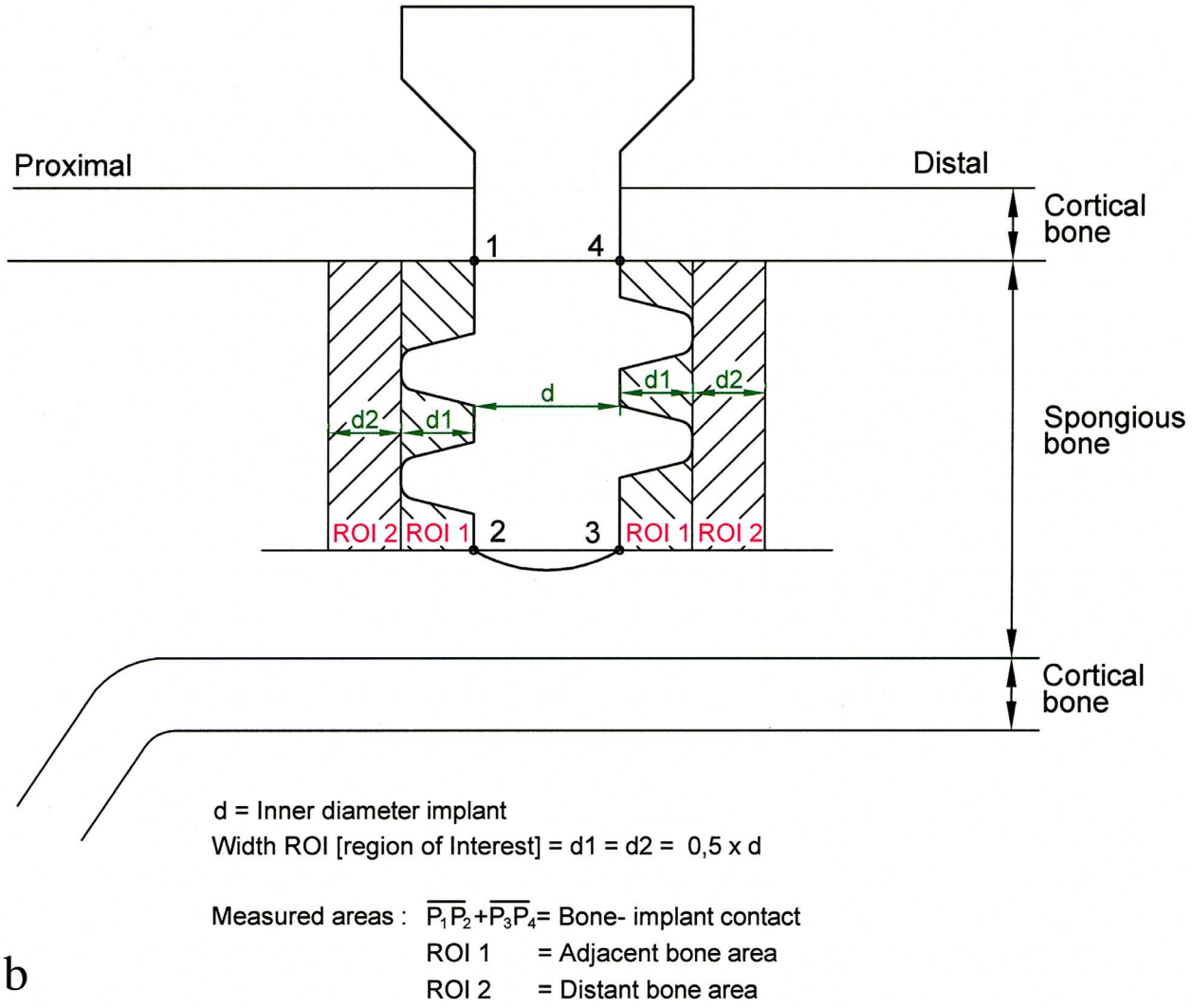
After sacrifice, the left tibiae were harvested. The proximal halves were fixed in Schaffer`s solution for 24 h at 4 °C, followed by 70 % ethanol. Following *ex vivo* microCT analysis, the samples were dehydrated and embedded in methylmethacrylate to generate 50 µm ground sections cut in parallel to the implant length axis and perpendicular to the tibial axis as described previously (Donath and Breuner, 1982). Fluorescent microscopy was performed prior to Masson-Goldner staining.

Computed tomography

For even distribution of animals into groups prior to implant surgery, cross-sectional apparent cancellous bone-mineral density (BMD) was evaluated in the left proximal tibia metaphysis by peripheral quantitative computed tomography (pQCT, Norland XCT-2000 Stratec, Pforzheim, Germany, fitted with an Oxford 50 AM X-ray tube and a collimator of 1 mm diameter; voxel size: 0.2 x 0.2 x 1 mm; scan speed: scout view 20 mm/s; final scan 10 mm/s, 1 block, contour mode 1, peel mode 2; threshold: 610 mg/cm^3).

Ex vivo CT measurements were performed with a microCT40 (Scanco Medical, Brüttisellen, Switzerland; voxel size, 10 µm; high resolution; 192 slices, energy, 70 E(kVp), 114 µA, high resolution, integration time 300 ms, conebeam continuous rotation) as previously described (Rebaudi *et al.*, 2004). Briefly, bone and titanium implant were distinguished using the appropriate Gaussian filters (sigma 1.2 bone, 2.0 titanium; support 2 bone,





Fig, 1. Regions of interest for (**a**) micro-CT measurements and (**b**) histomorphometric measurements.

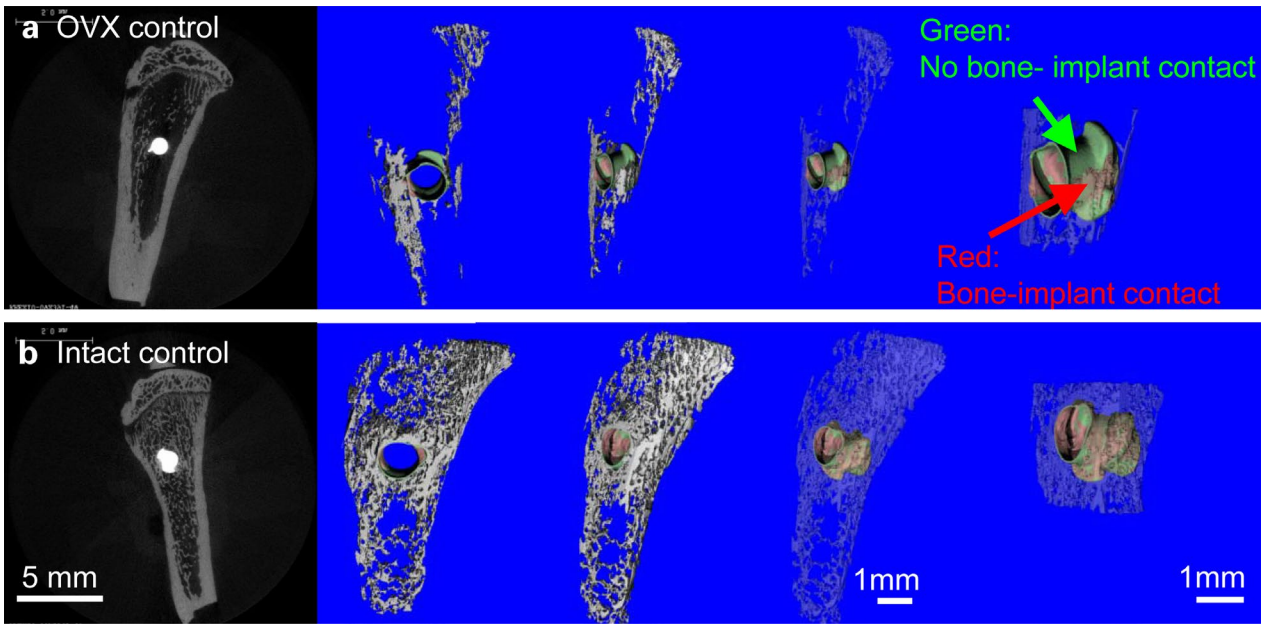


Fig. 2. MicroCT images exemplifying the difference in bone template in the proximal tibia between oestrogen competent (**a**) and oestrogen deprived (**b**) animals four weeks post-implantation.

3 titanium) and threshold procedures (bone: 370-700; titanium implant 700-1000). BMD and bone volume/tissue volume (BV/TV) were determined in the cancellous bone region surrounding the implant (3 to 10 voxels distance from the implant) and in a neighbouring region in further distance to the implant surface (10 to 23 voxels distance from the implant) (Fig. 1a). Since results did not differ between the two regions, data were merged for the results presented in Table 2 and reflect a total width of 200 µm. In addition, bone-implant contact (BIC) was determined. In order to avoid artefacts due to the titanium implant, BIC measurements were performed at a distance of 30 µm (i.e., 3 voxels) from the surface. The number of surface voxels attached to bone at this distance divided by the total number of implant surface voxels was used to derive the BIC in percentage, as described by Rebaudi *et al.* (2004).

Histomorphometry

Fluorescence microscopy was performed at up to 40x magnification (Olympus BX 61, Hamburg, Germany). Polyfluorochrome labels were qualitatively analysed for bone growth dynamics, location and label sequence. Subsequently stained sections were imaged using a motorised measuring stage (Märzhäuser, Wetzlar, Germany) for multiple alignment scanning connected to a computerised system of histomorphometry (CellˆF, Imaging Software for Life Science, Olympus). BIC was measured in the cancellous bone compartment along the implant surface. The apex of the implant was not included. Cancellous bone area per tissue area (BA/TA) was measured in a region adjacent to the implant and in a surrounding distant area (Fig. 1b).

Biomechanical evaluation

All specimens underwent biomechanical testing at the day of harvest. Healing caps were removed and the tibiae were embedded in dental plaster (Fujirock EP, Improved type 4 dental stone, GC Europe, Kortrijk, Belgium), using a custom potting device which provides coaxial alignment of the implant and the testing machine. Specimens were mounted on a servohydraulic testing machine (MTS 858 Mini Bionix, MTS Systems, Eden Prairie, MN, USA) and connected to a 2 Nm load cell/signal amplifier (D2209, Lorenz Messtechnik, Alfdorf, Germany, accuracy 2 Nmm). A mechanical flex clutch was used to ensure neutral initial fixation (no moment). Implants were rotated counter-clockwise (CCW) at a constant rate of 0.5°/s. Moment and angle data were recorded for subsequent analysis with a custom script in MATLAB (R2008, The MathWorks, Natick, MA, USA) to determine the removal torque (RT: Nmm) and interfacial stiffness (Nmm/°) values.

Statistical analysis

The data are presented as mean values plus/minus standard deviation. Data distribution was tested by the Kolmogorov-Smirnov-Test. Variance analysis of all groups by analysis of variance (ANOVA) and following Bonferoni adjusted multiple comparisons of mean values were performed. Differences in variance between measurement parameters were analysed by Pearson correlation coefficient. The level of significance was set at $\alpha = 0.05$ in all statistical tests. Statistical analysis was performed by SPSS for Windows® 19 (SPSS, Chicago, IL, USA).

Results

Animals

Six animals died during anaesthesia and one animal was euthanised due to wound healing complications. Seven histological samples were not included in the histological analysis due to bicortical insertion.

Table 3. Results of histomorphometry at two and four weeks.

Group	Surface	Healing time		BIC cancellous bone		BA adjacent		BA distant	
		[weeks]	*n*	[%]	SD	[%]	SD	[%]	SD
non-OVX	reference	2	7	52.9#	14.6	47.1#	19.9	28.0#	13.6
	reference	4	6	66.5#	11.5	42.6#	11.8	29.3#	11.2
	conditioned	2	7	55.3#	13.6	43.1#	12.7	28.3#	9.7
	conditioned	4	7	51.1#	12.0	43.0#	13.5	30.6#	10.0
OVX	reference	2	8	24.1*	8.6	16.4*	9.0	8.7*	6.7
	reference	4	7	10.7*	9.3	4.5*	4.2	6.0*	5.9
	conditioned	2	7	35.2	18.0	16.0*	6.5	10.2*	5.8
	conditioned	4	6	19.3*	6.8	6.3*	3.7	8.6*	4.6
	collagen/CS	2	8	31.5	12.2	18.4*	7.5	10.0*	6.6
	collagen/CS	4	7	14.2*	8.0	5.6*	4.2	5.7*	2.7
	simvastatin	2	8	38.9	19.8	28.6*	9.9	17.7	4.7
	simvastatin	4	6	25.6*	4.6	9.3*	3.9	6.0*	3.4
	zoledronic acid	2	7	18.1*	8.4	23.1*	6.2	9.7*	7.0
	zoledronic acid	4	7	43.2*#	17.8	23.8*#	8.6	7.3*	5.4

Significantly different ($p \leq 0.05$) compared to reference in non- OVX (*) or OVX (#) rats at the same time point; equal number of animals (*n*) for all parameters.

Bone-Implant Contact (BIC)

BIC was comparable between the oestrogen competent groups having received the reference implant or the implant with the conditioned surface both at two and four weeks according to microCT and histomorphometric readouts (Tables 2 and 3). The two corresponding OVX groups were also comparable at both time points. However, they had dramatically reduced BIC compared to intact animals. Structurally these findings correlated to a poor bone template in the long-term oestrogen deprived animals: both in the region adjacent to the implant and the neighbouring region (Tables 2 and 3, Fig. 2).

Two weeks post-implantation neither collagen/chondroitin sulphate nor zoledronic acid coating had any impact on BIC compared to OVX controls (Tables 2 and 3), while statin coating improved BIC. After four weeks however, no effect related to latter was detectable. At this time point zoledronic acid coated implants displayed a higher BIC than controls, while the BIC of implants having received collagen/chondroitin sulphate coating remained comparable to controls (Tables 2 and 3). Consequently, all oestrogen-deprived osteopenic animals displayed lower BIC than oestrogen-competent animals with the exception of those having received zoledronic acid coated implants. In general BIC was lower in OVX rats after four weeks compared to two weeks, with the exception of the group with zoledronic acid coated implants which displayed a higher BIC at the later time point (Tables 2 and 3).

Cancellous bone mass

MicroCT analyses demonstrated dramatically reduced cancellous bone volume (BV/TV) and cancellous bone-mineral density (BMD) in all oestrogen-depleted animals two and four weeks post-implantation compared to oestrogen-competent rats irrespective of implant surface treatment (Table 2). However, BV/TV was higher in the group having received zoledronic acid coated implants compared to all other OVX groups after four weeks (Table 2). BMD values showed a similar pattern. Analogous to BIC, the amount of cancellous bone decreased in OVX rats between two and four weeks with the exception of the group having received zoledronic acid coated implants, while it remained stable in intact animals (Table 2).

Histomorphometric results (Table 3) confirmed the microCT findings. Oestrogen deprived rats had at both time points irrespective of implant surface treatment lower bone area (BA) values adjacent to the implant. Consistently BA was higher in the group having received zoledronic acid coated implants compared to all other OVX groups after four weeks (Table 3). Moreover, in alignment with the microCT results BA adjacent to the implant decreased in OVX rats between two and four weeks with the exception of the group having received zoledronic acid coated implants, but was stable in intact animals (Table 3). The distant BA neighbouring the tissue area adjacent to the implant showed a slightly different pattern, insofar as all OVX groups displayed at both time points reduced BA compared to non-OVX animals including the zoledronic acid coated implant group with one exception. At the two-week time point, the group which had been exposed to statin coated implants displayed in this region a higher BA (Table 3). However, by four weeks this effect was not detectable anymore and BA was comparable between OVX groups, which was somewhat lower, though not

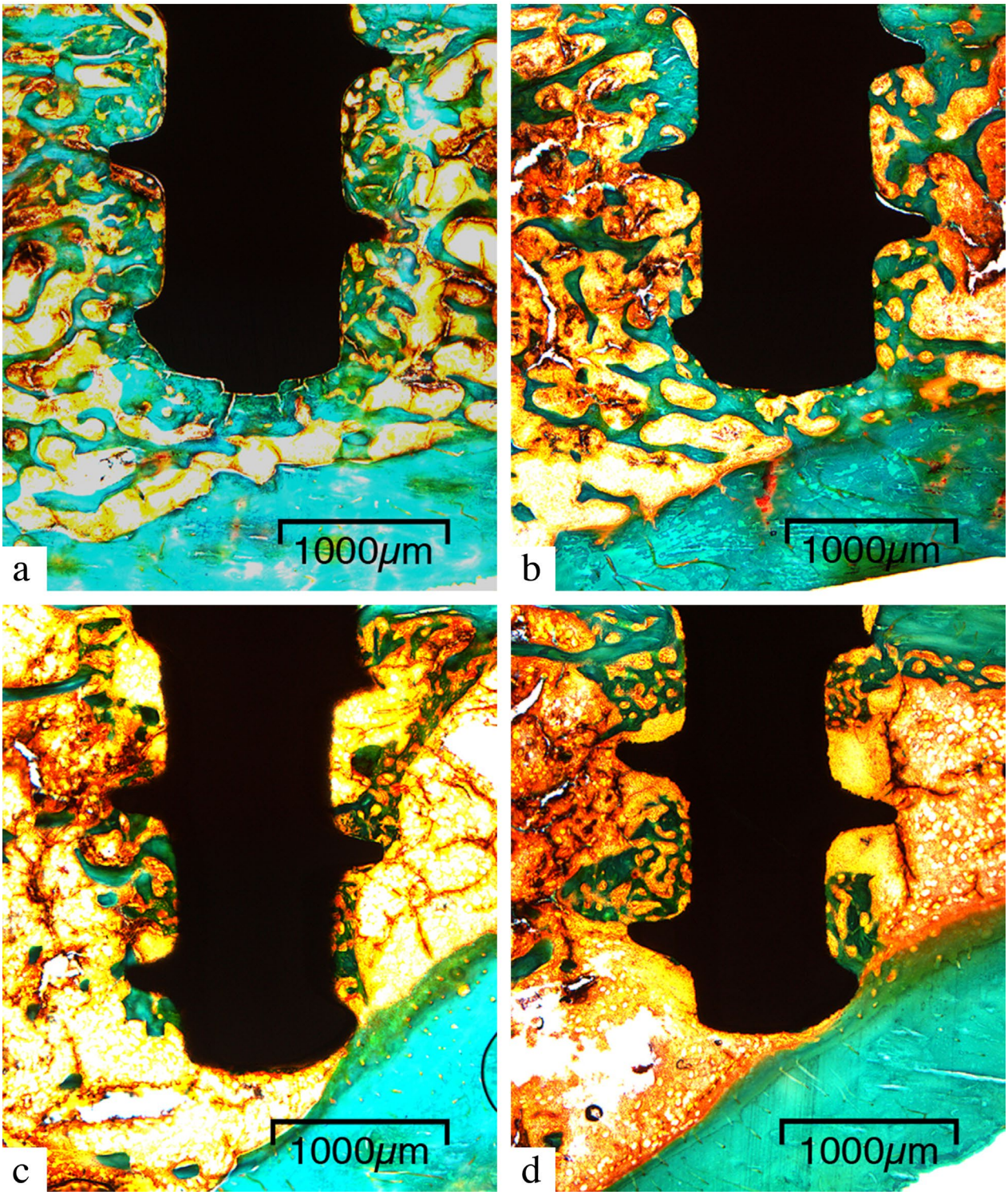


Fig. 3. Histology (Masson-Goldner staining): After 2 weeks, reference implant surfaces in the tibiae of oestrogen competent rats displayed homogenous bone formation within implant threads (**a**). This zone was remodelled by 4 weeks and the implant integrated in the regular trabecular bone structure (**b**). The tibiae of OVX rats with zoledronic acid coated implants; also some trabecular structures with partial implant contact were visible after 2 weeks (**c**), which were also observed after 4 weeks within implant threads (**d**).

significantly so (with the exception of the statin exposed group), than at two weeks. Overall, BA was higher in the area adjacent to the implant than in the neighbouring distant region (Table 3, Fig. 3).

Fluorochrome marker uptake

Calcein labels were readily detectable and more pronounced two weeks post-implantation. In addition to the incorporation of fluorochrome labels in the unaffected secondary spongiosa and endosteum at sites of bone formation and mineralisation related to normal bone turnover, a high level of intensive calcein labelling was detectable in peri-implant tissues up to 1 mm distance from the implant surface. This appeared most pronounced for the collagen/chondroitin sulphate and statin coated surfaces. By four weeks peri-implant label uptake was low. Alizarin,

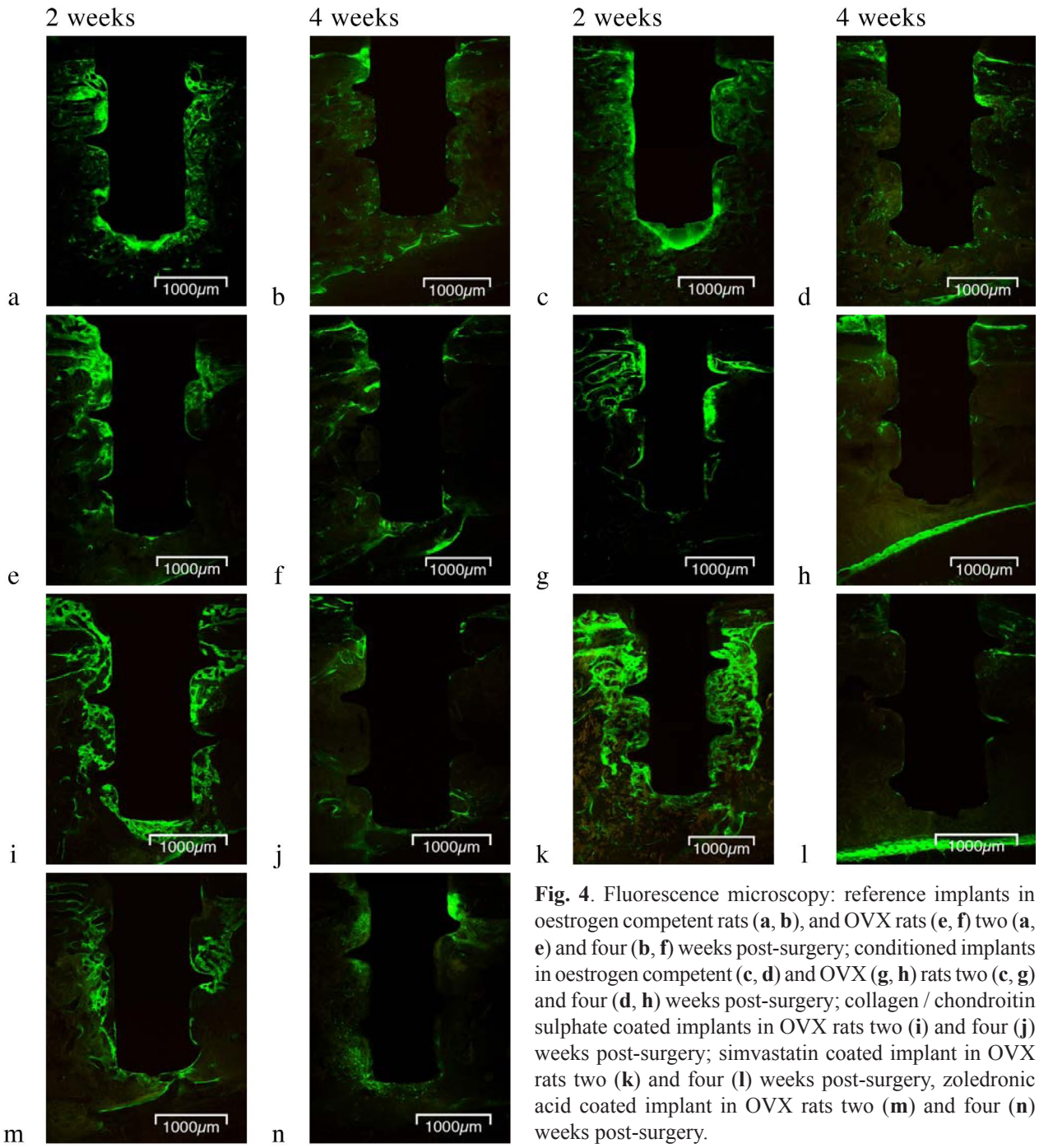


Fig. 4. Fluorescence microscopy: reference implants in oestrogen competent rats (**a**, **b**), and OVX rats (**e**, **f**) two (**a**, **e**) and four (**b**, **f**) weeks post-surgery; conditioned implants in oestrogen competent (**c**, **d**) and OVX (**g**, **h**) rats two (**c**, **g**) and four (**d**, **h**) weeks post-surgery; collagen / chondroitin sulphate coated implants in OVX rats two (**i**) and four (**j**) weeks post-surgery; simvastatin coated implant in OVX rats two (**k**) and four (**l**) weeks post-surgery, zoledronic acid coated implant in OVX rats two (**m**) and four (**n**) weeks post-surgery.

which had been applied earlier than calcein, was generally rarely visible in the peri-implant area at either time point. A marked qualitative difference in peri-implant fluorochrome marker uptake was not noticed between OVX rats and intact controls. (Fig. 4).

Biomechanical properties

Removal torque was lower in all oestrogen-deprived groups compared to oestrogen-competent groups (Table 4) at both time points. None of the implant surface coatings had any significant impact on removal torque at either time point (Table 4). Removal torque was higher at four weeks in all groups compared to two weeks, reaching significance in the intact groups and the OVX groups exposed to zoledronic acid or collagen/chondroitin coated implants (data not shown). Stiffness was largely comparable between groups irrespective of oestrogen status and implant surface treatment (Table 4). Only the OVX group having received the reference implant displayed decreased stiffness at the two-week time point. Generally, stiffness was somewhat higher at four weeks in all groups reaching significance in most with the exception of the intact group with the conditioned implant surface and the OVX group with the statin coated surface (data not shown).

Correlation between assessed parameters

Consistent with the above-described findings the correlation analysis showed a significant correlation between various parameters two and four weeks post-implantation: BIC by microCT and histomorphometry showed a significant correlation to adjacent and distant BA by histomorphometry, BV/TV and BMD by microCT and

Table 4. Results of mechanical testing at two and four weeks.

Group	Surface	Healing time		Removal torque		Stiffness	
		[weeks]	*n*	[Nmm]	SD	[Nmm/°]	SD
non-OVX	reference	2	8	57.0#	13.9	29.5#	2.5
	reference	4	8	85.7#	21.9	31.3	3.4
	conditioned	2	8	56.0#	18.6	29.5#	3.6
	conditioned	4	8	94.7#	20.9	35.3	4.4
OVX	reference	2	8	30.5*	9.6	19.2*	10.1
	reference	4	8	41.5*	15.5	33.3	8.2
	conditioned	2	8	36.7	12.5	26.1*	7.2
	conditioned	4	8	43.6*	7.5	33.7	5.6
	collagen/CS	2	8	37.4	13.0	26.5	3.9
	collagen/CS	4	8	50.4*	9.8	36.7	6.9
	simvastatin	2	8	32.0*	12.6	22.1	7.1
	simavstatin	4	8	44.2*	13.6	28.5	7.1
	zoledronic acid	2	8	29.8*	10.5	24.1	6.0
	zoledronic acid	4	8	52.9*	17.0	31.0	4.3

Significantly different ($p \leq 0.05$) compared to reference in non- OVX (*) or OVX (#) rats at the same time point; equal number of animals (n) for both parameters.

removal torque. The results of the stiffness test correlated to removal torque at both time points and 2 weeks post-implantation to BV/TV and BMD (Table 5).

Discussion

Several previous studies have analysed the impact of conditioning or drug coating of implant surfaces in animal models of osseointegration (Buser *et al.*, 2004; Rammelt *et al.*, 2007; Wermelin *et al.*, 2008b). Increased peri-implant bone formation and improved biomechanical characteristics have been demonstrated for such surface treatments in some cases. An improvement of osseointegration would be especially beneficial in compromised osseous situations such as osteoporosis (Bernhardt *et al.*, 2005), which is characterised by a systemic deterioration of bone mass and microarchitecture (Rachner *et al.*, 2011). Studies analysing implant osseointegration in osteoporotic patients yielded differential results. Whereas some studies report increased implant loss rates for osteoporotic patients (Moy *et al.*, 2005; Alsaadi *et al.*, 2007), others did not observe increased implant loss rates for these patients (Dvorak *et al.*, 2011). A systematic review describes the level of evidence for studies on this matter as multiple case-control studies (Bornstein *et al.*, 2009). In the present study, we analysed the osseointegration of a number of different conditioned or coated implants. Some of these surfaces have previously been described to display increased osseointegration in animal models under compromised osteoporotic conditions. We utilised aged rats, which were oestrogen-deprived by ovariectomy, as a standard model for the simulation of post-menopausal osteoporosis (Thompson *et al.*, 1995). Following ovariectomy, bone resorption exceeds bone formation, increasing bone turnover and inducing rapid cancellous bone loss resulting in a severely rarefied trabecular network within three months (Jee and Yao, 2001). We placed the implants three months post-ovariectomy into the proximal tibia metaphysis as a well characterised skeletal site for sex-hormone deprival induced bone loss. As expected, both microCT and histological evaluation confirmed a severely compromised cancellous bone network in the proximal tibia of those aged animals.

The first implant modification for improvement of implant osseointegration we tested was surface conditioning as a means to modify the physicochemical characteristics of an implant. Histomorphometric and micro-CT measurements of BIC and bone volume served to quantify peri-implant bone formation. The correlation of histological and micro-computed tomographic data for the analysis of BIC and bone volume has been analysed in an earlier study applying synchrotron radiation (Bernhardt *et al.*, 2012). The conditioning by hydroxide ions influences surface charge, hydrophilicity and the homogeneity of the initial protein layer (Tugulu *et al.*, 2010). Various animal studies utilising non-osteoporotic conditions have analysed mechanics and histomorphometry of such conditioned implants, and demonstrated increased bone formation and removal torque values indicating improved osseointegration (Ferguson *et al.*, 2006; Calvo-Guirado *et al.*, 2010; Stadlinger *et al.*, 2012). However, in our setting, we observed – neither in osteoporotic oestrogen deprived nor in non-osteoporotic oestrogen-competent animals – an increase of bone-implant contact or an improvement of biomechanical properties in comparison to reference

Table 5. Inter-group-correlation analysis. Pearson correlation coefficient and two-tailed *p* values (in brackets) are shown for each parameter and time point.

Time	Parameter	BIC (Hist.)	BA adjacent	BA distant	Removal torque	Stiffness	BIC (MicroCT)	BV/TV	BMD
2 weeks	BIC (Hist.)	1	0.685**	0.638**	0.382**	0.224	0.538**	0.476**	0.473**
			(0.000)	(0.000)	(0.005)	(0.111)	(0.000)	(0.001)	(0.001)
	BA adjacent	0.685**	1	0.910**	0.406**	0.153	0.617**	0.617**	0.616**
		(0.000)		(0.000)	(0.003)	(0.278)	(0.000)	(0.000)	(0.000)
	BA distant	0.638**	0.910**	1	0.318*	0.132	0.687**	0.652**	0.655**
		(0.000)	(0.000)		(0.021)	(0.351)	(0.000)	(0.000)	(0.000)
	Removal torque	0.382**	0.406**	0.318*	1	0.601**	0.490**	0.521**	0.507**
		(0.005)	(0.003)	(0.021)		(0.000)	(0.001)	(0.000)	(0.000)
	Stiffness	0.224	0.153	0.132	0.601**	1	0.275	0.353*	0.294*
		(0.111)	(0.278)	(0.351)	(0.000)		(0.067)	(0.012)	(0.036)
	BIC (MicroCT)	0.538**	0.617**	0.687**	0.490**	0.275	1	0.962**	0.973**
		(0.000)	(0.000)	(0.000)	(0.001)	(0.067)		(0.000)	(0.000)
	BV/TV	0.476**	0.617**	0.652**	0.521**	0.353*	0.962**	1	0.985**
		(0.001)	(0.000)	(0.000)	(0.000)	(0.012)	(0.000)		(0.000)
	BMD	0.473**	0.616**	0.655**	0.507**	0.294*	0.973**	0.985**	1
		(0.001)	(0.000)	(0.000)	(0.000)	(0.036)	(0.000)	(0.000)	
4 weeks	BIC (Hist.)	1	0.847**	0.700**	0.570**	-0.056	0.807**	0.764**	0.786**
			(0.000)	(0.000)	(0.000)	(0.714)	(0.000)	(0.000)	(0.000)
	BA adjacent	0.847**	1	0.893**	0.615**	0.029	0.867**	0.817**	0.844**
		(0.000)		(0.000)	(0.000)	(0.849)	(0.000)	(0.000)	(0.000)
	BA distant	0.700**	0.893**	1	0.600**	0.094	0.764**	0.765**	0.774**
		(0.000)	(0.000)		(0.000)	(0.536)	(0.000)	(0.000)	(0.000)
	Removal torque	0.570**	0.615**	0.600**	1	0.316*	0.614**	0.695**	0.703**
		(0.000)	(0.000)	(0.000)		(0.018)	(0.000)	(0.000)	(0.000)
	Stiffness	-0.056	0.029	0.094	0.316*	1	-0.085	-0.079	-0.070
		(0.714)	(0.849)	(0.536)	(0.018)		(0.568)	(0.584)	(0.630)
	BIC (MicroCT)	0.807**	0.867**	0.764**	0.614**	-0.085	1	0.970**	0.978**
		(0.000)	(0.000)	(0.000)	(0.000)	(0.568)		(0.000)	(0.000)
	BV/TV	0.764**	0.817**	0.765**	0.695**	-0.079	0.970**	1	0.992**
		(0.000)	(0.000)	(0.000)	(0.000)	(0.584)	(0.000)		(0.000)
	BMD	0.786**	0.844**	0.774**	0.703**	-0.070	0.978**	0.992**	1
		(0.000)	(0.000)	(0.000)	(0.000)	(0.630)	(0.000)	(0.000)	

* The correlation was statistically significant at a level of 0.05 (two tailed). ** The correlation was statistically significant at a level of 0.01 (two tailed).

surfaces. This difference might relate to a diminishing pool of osteoprogenitor cells in those ageing animals (Pei *et al.*, 2003). Next, we tested whether implant coating with components of the bone extracellular matrix (ECM) would stimulate bone osseointegration in our compromised setting (Stadlinger *et al.*, 2009a). Collagen type I is the main structural component of the bone organic ECM and has been previously demonstrated to favour the adhesion of osteoblastic cells (Becker *et al.*, 2002). In addition, the application of glycosaminoglycans such as chondroitin sulphate (CS) influences the adhesion, proliferation and differentiation of osteoblasts (Bierbaum *et al.*, 2006; Douglas *et al.*, 2007). Accordingly, the suitability of collagen/CS coated implant surfaces has been assessed in various *in vitro* and *in vivo* studies. In minipigs, collagen/CS coated implants showed improved implant osseointegration, compared to pure collagen coatings (Stadlinger *et al.*, 2008). Such coated implants, displayed in mandibular and maxillary bone, increased BIC compared to sandblasted, acid-etched implants 4 weeks post-implantation (Stadlinger *et al.*, 2009a; Stadlinger *et al.*, 2011). In addition, Rammelt *et al*. examined collagen/CS coated intramedullary nails in tibiae of 90 days old intact male rats and showed increased bone formation and bone

remodelling in comparison to uncoated surfaces (Rammelt *et al.*, 2006). In our study in older female rats, collagen/CS coated surfaces did not show significant differences in mechanics and peri-implant bone formation in comparison to uncoated surfaces. Increased values in mechanical tests did not reach a level of significance. This is somewhat unexpected as the promising results obtained previously in large animal models suggested that a pronounced positive effect might also be detectable under osteoporotic conditions. Yet, our results indicate that in a setting of severely reduced bony template and a presumably reduced osteoblastic precursor pool such surface coatings cannot exert their beneficial effect.

We also tested a principle that has been previously suggested to impact bone formation by stimulating bone morphogenetic proteins (BMPs). It is well established that the differentiation of osteoblastic cells is increased by BMPs (Mundy *et al.*, 1999) and that this is relevant to implant osseointegration. Different studies have suggested that BMP expression can be stimulated by statins (Oxlund *et al.*, 2001; Skoglund *et al.*, 2002). Clinically, statins are administered to decrease high cholesterol levels through inhibition of the enzyme HMG-CoA reductase which is involved in the cholesterol synthesis. Induction of an osteoanabolic effect occurs only at higher dosages than required for cholesterol inhibition (Moriyama *et al.*, 2010). In addition, a putative inhibition of osteoclast activation has been proposed (Moriyama *et al*., 2010). Statins have been applied in various animal models of osseointegration and improved implant integration rates in non-compromised (Ayukawa *et al.*, 2010; Moriyama *et al.*, 2010) and osteoporotic animal models (Du *et al.*, 2009) have been reported. Local application of fluvastatin increased peri-implant bone formation in the tibial bone of oestrogen competent growing ten-week-old female rats, (Moriyama *et al.*, 2010). In addition, systemic high dose treatment improved peri-implant bone deposition in the implant carrying tibial medullar cavity of intact skeletally mature 30-week-old female rats (Ayukawa *et al.*, 2010). Moreover, systemic application of simvastatin to 5-month-old rats, which had been ovariectomised two months prior to implant placement, also enhanced osseointegration (Du *et al.*, 2009). We used in our study older animals than any previous study and placed implants after a more extended oestrogen deprival induced bone loss period, while the examined implant healing intervals were comparable to other studies. In comparison to Du *et al.* (2009), cancellous bone loss was far more pronounced in our study. In addition, implant insertion was in the present study mono-cortical while Du *et al*. performed bi-cortical insertion. Since the successful local application of statins has been demonstrated to depend on an effective drug delivery system, like chitosan, PGA (Moriyama *et al.*, 2010) or methylcellulose gels (Thylin *et al.*, 2002), we have utilised chitosan in the present study. In accordance with previous studies, we observed a transient increase in BIC and the amount of peri-implant bone in the group that had received statin coated implants. Consistently, fluorochrome marker uptake appeared in the peri-implant area elevated two weeks post-implantation compared to other surfaces. However, these changes did not translate into improved biomechanical readouts and at the later time-point no impact of the statin coating was detectable any more. Since we consider our animal model to more faithfully mimic severely compromised osteoporotic settings, we conclude that the ability of locally released statins to improve osseointegration under such conditions is limited.

Finally, to complete the spectrum of implant surface modifications that are known to impact bone metabolism and thus may impact osseointegration we tested an anti-resorptive principle. Bisphosphonates are potent anti-resorptive drugs that primarily target osteoclasts (Abtahi *et al.*, 2010). Clinically, bisphosphonates are administered intravenously in patients suffering from bone metastasis of malignant tumours. Lower doses of various bisphosphonates are applied by infrequent oral or intravenous injection for various bone fragility conditions including osteoporosis. In addition to systemic treatments, local release from implant surfaces has already been tested previously in preclinical settings (Gao *et al.*, 2009; Wermelin *et al*., 2008b). Zoledronic acid is a potent bisphosphonate with a high affinity to mineralised bone (Li and Davis, 2003). In the present study zoledronic acid coated surfaces were the only implants that lead to a significant increase in BIC from two to four weeks. The animals that had received those implants displayed an increase in the amount of bone surrounding the implant at the end of the study. Consistently, maintenance of a higher amount of fluorochrome labelled peri-implant bone was visible at the end of the study compared to other groups. Since bone resorption and bone formation are uncoupled during a repair process like osseointegration, bone formation can commence despite the presence of the anti-resorptive bisphosphonate opposed to situations in which bone resorption and bone formation are coupled such as normal adult bone remodelling (Baron and Kneissel, 2013). However, the apparently increased fluorochrome marker uptake does not necessarily reflect increased bone formation compared to the other groups, but could simply reflect the maintenance of the newly formed bone by anti-resorptive action, while this bone was rapidly removed in the other groups in the absence of such a principle. As mentioned above, various previous studies analysed the application of bisphosphonates on osseointegration in animals. Consistent with our results, improvement of the amount of peri-implant bone formation was also detected in OVX rats after 8 weeks (Gao *et al.*, 2009). Of note compared to our study, Gao *et al*. operated younger rats, which however also underwent implant placement in the tibial medullar cavity three months post-OVX. Three months post-implantation, bone area and BIC were found to be doubled in animals carrying implants with bisphosphonate coatings on a hydroxyapatite implant surface layer (Gao *et al*., 2009). Our findings are overall consistent with other studies, showing increased implant fixations for bisphosphonate coatings using different carriers and displaying different drug release kinetics (Andersson *et al.*, 2010; Peter *et al.*, 2006), though involved. Wermelin *et al*. analysed the surface release of bisphosphonates, showing that one third of the coating was released within 4 weeks (Wermelin *et al.*, 2008a). Gao *et*

al. demonstrated zoledronic acid coating up to 21 days after implantation (Gao *et al.*, 2009). In the present study, analyses of the release kinetics of statin and zoledronic acid coatings revealed a surface release restricted to 7-13 days, indicating that this local relatively short-term release was sufficient to induce longer-term effects as detected up to one month. Though the bisphosphonate coating emerged in this setting of severely osteoporotic and compromised bone as the most promising principle, safety aspects have to be considered. Clinically, the intra-oral application of bisphosphonates needs to address the aspect of oral necrosis of the jaw (ONJ). The ONJ has been described in various studies for the systemic application of high dose bisphosphonates in cancer patients (Marx, 2003; Ruggiero *et al.*, 2009). Abtahi *et al*. addressed this question and describes that no animal study observed to date necrotic bone around bisphosphonate coated implants (Abtahi *et al.*, 2010). They further examined the infiltration depth of surface bound bisphosphonates into the peri-implant bone, measuring a maximum distance of less than 1 mm (Abtahi *et al.*, 2010; Wermelin *et al.*, 2008b). A translation of the mentioned animal studies to the human situation is limited by the fact that implants healed submerged. A loaded dental implant in humans, however, is exposed to the oral cavity and oral bacteria. A further consideration is long-term implant stability, which requires continuous bone remodelling in order to preserve osseointegration (Roberts *et al.*, 1992). To date, first results in humans receiving zoledronic acid coated implants indicate increased stability after 6 months of submerged healing (Abtahi *et al.*, 2012). Further investigations are required to assess how implants from which bisphosphonates have been released respond to remodelling processes following implant loading. Limiting release of the drug to a shorter time window, like in the present study, may be one way to limit the amount of peri-implant bone that is exposed to the anti-resorptive principle, allowing hence for a faster recovery of remodelling based bone turnover.

In summary, the present study assessed the impact of implant surface modifications, which have resulted previously in improved osseointegration in preclinical settings, in an animal model of severe osteoporosis. In this setting, most modifications did not yield an appreciable improvement, though simvastatin coating had a transient positive impact on the amount of peri-implant bone and the amount of bone-implant contact. Only zoledronic acid coating improved osseointegration at the end of the study, suggesting that such coatings may be of interest in the settings of severely compromised bone template. Further studies are, however, required to demonstrate the long-term success of such coated implants.

Acknowledgements

This study was funded by Thommen Medical AG. The authors thank Peter Ingold, Marcel Merdes, Marco Pegurri, Renzo Schumpf, Andrea Venturiere, and Anne Studer for their contribution to this study. We also thank the project partners of the SFB Transregio 67 of the DFG (German Research Foundation), especially projects A3 and A2.

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Discussion with Reviewers

Reviewer I: Which animal model would you prefer if you would repeat the experiment in a large animal model? What differences to the presented approach would you expect? Which size of implants would you choose? The larger size of implants would lead to an increased load of bioactive factors. Could this improve results?
Authors: A possible large animal model would be minipigs. Ovarectomy-induced oestrogen deficiency has been shown to manifest stronger in primiparous as compared to nulliparous minipigs (Scholz-Ahrens *et al.,* 1996, additional reference). Supplementary calcium deficient diet and glucocorticoid application, however, has been able to induce osteopenia in nulliparous (Scholz-Ahrens *et al.,* 2007, additional reference). The advantage would be the placement of clinically applied implants. Larger implants of course would offer the opportunity to increase the load of bioactive factors. However, the effect of higher drug doses on osseointegration has to be carefully tested, Based on previous reports in the literature (Peter *et al*., 2006, text reference), there seems to be an optimal dosage, especially for the use of bisphosphonates.

Reviewer II: The aim of this work was to assess the impact of surface modifications on implants osseointegration and anchorage in a severely osteoporotic rat model. The study suggests that most surface modifications that have a positive effect in healthy bone may not have it in osteoporotic bone anymore. It is surprising that an effect is seen in bone-implant contact and bone density around implants, but this doesn't translate into a mechanical benefit. Could the authors comment on this discrepancy?
Authors: Although the constituents of bone are present (as evidenced by the mineral content and the bone-implant-contact), the development of these constituents into a fully-functional and integrated composite is delayed or compromised in osteoporotic bone. This may be seen as analogous to the difference between immature woven bone and fully remodelled, functional bone.

Reviewer II: What was the rationale to choose removal tests rather than pull-out or shear resistance tests? Have the authors considered FE modelling to numerically estimate pull-out strength from the microCT data?
Authors: Removal torque testing of threaded implants is a reliable method for determining the influence of surface treatments on osseointegration, which is less sensitive to testing artefact. Push-out, pull-out or shear tests are either subject to end effects with axial loading into the bone, require a substantially-sized implant to facilitate pull-out testing, or require specimen sectioning to perform shear tests. Any cutting of the implant/bone interface, e.g. to produce a standard push-out or shear test specimen, brings with it the risk of damaging the bone-implant interface. In the rat model, the chosen implant and torque test have the advantage of providing a low-profile implant head, which requires no further specimen preparation before performing the biomechanical test. The entire bone can be mounted for testing and the implant must not be strongly clamped in the testing machine to perform the measurement; it requires only a form-fit adapter and therefore no mechanical load is placed on the implant prior to testing.

FEA based on microCT has been shown to be inaccurate in the prediction of interfacial mechanical behaviour, as the true nature of the interface (bonded or non-bonded) cannot be determined a priori and non-bonded interfaces are computationally expensive. Therefore, FE models based on microCT rarely provide an accurate prediction of interfacial stiffness or strength.

Reviewer III: If (especially a straight) implant is loaded with a specific coating, how can the authors be sure not to detach the coating while installing the implant screw, leading to a very high concentration in the upper part and only a very low concentration in the lower? The implant thread design looks very aggressive, would you expect the same results even with an implant with more shallow threads, leaving less space between the bone and the implant?

Authors: The selected implant thread design serves to enable primary implant stability in this reduced bone quality. In this animal model, more shallow threads may cause micromotion and thus impede a possible conclusion on surface coating effects. Due to the low concentration of agents, very thin layers below 5 µm thickness were produced. The applied coating method produces relatively homogenous layers that strongly attach to metal surfaces. Certainly mechanical friction during implant insertion will influence the coating especially in the tip areas. However, due to the prior implant surface modification by sandblasting and acid etching, the coating components also penetrate into surface pores, thus being secured from friction during implant placement. The demonstrated effects of coating agents demonstrate sufficient remaining agent on the surface.

Additional References

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